PB83-210294

Utilization of the Rabbit Alveolar Macrophage and Chinese Hamster Ovary Cell for Evaluation of the Toxicity of Particulate Materials. 1. Model Model Compounds and Metal-Coated Fly Ash

(U.S.) Health Effects Research Lab.
Research Triangle Park, NC

1981

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TECHNICAL REPORT DATA
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1. REPORT NO.	2.	3.
EPA-600/J-81-098	JOURNAL ARTICLE	PB83-210294

4. TITLE AND SUBTITLE	5. REPORT DATE
The Utilization of the Rabbit Alveolar Macrophage and Chinese Hamster Ovary Cell for Evaluation of the Toxicity of Particulate Materials I. Model Compounds and Metal-Coated Fly Ash	1981
	6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Neil Garrett, James Campbell, Frank Stack, Michael Waters Joellen Lewtas	
9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
Cellular Pathology/Biochemistry/Genetic Toxicology Health Effects Research Laboratory US Environmental Protection Agency Research Triangle Park, NC 27711	11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Office of Research & Development Health Effects Research Laboratory US Environmental Protection Agency Research Triangle Park, NC 27711	14. SPONSORING AGENCY CODE EPA-600/11

15. SUPPLEMENTARY NOTES

Published In: Environmental Research 24, 345-365, 1981

16. ABSTRACT

Data are presented which detail the effects of model particulate compounds and fly ash particles on rabbit alveolar macrophage (RAM) and Chinese hamster ovary (CHO) cells. Silica, silicic acid, titanium dioxide, and size-fractionated (0,-2, 2-5, and 5-8 um) fly ash particles with and without coatings of nickel, lead, or cadmium oxides were the experimental particles. Silica was the most toxic particle studied. Cell viability and ATP in the RAM assay and colony survival in the CHO assay showed an almost identical response to silica and silicic acid. Titanium dioxide particles were relatively inert in the RAM and CHO systems, although a pronounced loss of ATP was observed in cells exposed in serum-free medium. Uncoated fly ash was relatively nontoxic in the RAM system when assayed by measurement of cell number and viability, ATP, and total protein. However, toxicity of the uncoated particles was demonstrated in the CHO clonal assay. The number of colonies formed at 1000 μg/ml particulate was reduced to 10.6, 28.5, and 82.2% of the control for the 0 to 2 , 2 to 5, and 5 to 8 μm size ranges. Nickel oxide-coated fly ash was more cytotoxic than the uncoated particles in both the RAM and CHO cell systems.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to Public	Unclassified	22
	20. SECURITY CLASS (This Page) Unclassified	22. PRICE

EPA-600/J-81-098
JOURNAL ARTICLE

ENVIRONMENTAL RESEARCH 24, 345–365 (1981)

The Utilization of the Rabbit Alveolar Macrophage and Chinese Hamster Ovary Cell for Evaluation of the Toxicity of Particulate Materials

I. Model Compounds and Metal-Coated Fly Ash

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Received August 4, 1980

Data are presented which detail the effects of model particulate compounds and fly ash particles on rabbit alveolar macrophage (RAM) and Chinese hamster ovary (CHO) cells. Silica, silicic acid, titanium dioxide, and size-fractionated (0–2, 2–5, and 5–8 μm) fly ash particles with and without coatings of nickel, lead, or cadmium oxides were the experimental particles. Silica was the most toxic particle studied. Cell viability and ATP in the RAM assay and colony survival in the CHO assay showed an almost identical response to silica and silicic acid. Titanium dioxide particles were relatively inert in the RAM and CHO systems, although a pronounced loss of ATP was observed in cells exposed in serum-free medium. Uncoated fly ash was relatively nontoxic in the RAM system when assayed by measurement of cell number and viability, ATP, and total protein. However, toxicity of the uncoated particles was demonstrated in the CHO clonal assay. The number of colonies formed at 1000 μg/ml particulate was reduced to 10.6, 28.5, and 82.2% of the control for the 0 to 2-, 2 to 5-, and 5 to 8-μm size ranges. Nickel oxide-coated fly ash was more cytotoxic than the uncoated particles in both the RAM and CHO cell systems. Toxicity of NiO was similar to that obtained for the NiO-coated fly ash although the weight percentage of NiO in the ash was only 3%, suggesting that the particles enhanced toxicity. The lead oxide-coated fly ash was even more toxic than the nickel-coated particles; these particles were used to explore the effect of serum concentration on toxic responses. Cellular ATP was strongly affected in macrophages exposed in serum-free media and treated with PbO-coated fly ash; ATP ranged from 20 to 200 times less than that for the corresponding uncoated fly ash or untreated control. Nickel and lead did not dissociate from the fly ashes into the biological media. However, cadmium was rapidly released from the cadmium oxide-coated fly ash and provided an excellent model for study of the dissociation of toxic compounds from particle surfaces. The rate of dissociation of the metal was correlated with loss of ATP in RAM cultures. Cell numbers were unaltered after treatment with the CdO-coated fly ash as reported previously for soluble cadmium. The CdO-coated fly ash was considerably more toxic than would have been predicted on the basis of the amount of soluble cadmium released into the medium, also indicating that the association of the metal oxide with the fly ash enhanced toxicity.

INTRODUCTION

Airborne particles have been implicated in the increased incidence of asthma, pneumonia, bronchitis (Milby and Wolfe, 1969; Newball and Brahim, 1976) and

bronchogenic carcinoma (Berry *et al.*, 1972; Newball and Brahim, 1976; Matischnig *et al.*, 1977). The lung is a sensitive target for particulate matter and the bronchioalveolar region is particularly susceptible since the mucosal lining is covered with only a thin layer of surfactant (Witschi, 1975). In this region, the pulmonary alveolar macrophage phagocytizes particulate matter (Green, 1968, 1969).

Because of the key role of the alveolar macrophage in pulmonary defense, this cell type may be particularly important in the evaluation of the hazards of environmental particles. The rabbit alveolar macrophage (RAM) system has previously been shown to be sensitive to a variety of environmental contaminants (Waters *et al.*, 1974, 1975a, b; Mahar, 1976; Aranyi *et al.*, 1977, 1979; Huisingh *et al.*, 1977; Campbell *et al.*, 1978a, b; Garrett *et al.*, 1979). In addition, the Chinese hamster ovary (CHO) cell system has been shown to mimic the response of the macrophage for a variety of particulate materials (Garrett *et al.*, 1979, 1981). CHO cells are well characterized, and exhibit the useful property of forming discrete cell colonies. These cells are also capable of phagocytizing environmental particulate matter (Garrett *et al.*, 1979) and are useful in studies of mutagenicity and toxicity (Hsie et al., 1978; Wininger *et al.*, 1978, Garrett *et al.*, 1980).

The purpose of this paper is to describe the utilization of the RAM and CHO bioassay systems for the evaluation of the cytotoxicity of model particulate materials including chemically well-defined particulate substances and model fly ash.

MATERIALS AND METHODS

Sample Handling and Sources of Materials

Polystyrene latex particles were obtained from Dow Corning. Cab-O-Sil was from Pressure Scientific Co. Titanium dioxide particles were obtained from Polysciences. Silicic acid was from Fisher Scientific. Fly ash particles with and without coatings of specific metal oxides were obtained from the Fine Particle Division of the Illinois Institute of Technology Research Institute. All particulate samples were stored in desiccators containing Drierite to prevent absorption of moisture.

Short-Term Bioassay Method

The samples were evaluated using the RAM and CHO cell systems. Macrophages were obtained from male and female New Zealand white rabbits. The CHO cell line was obtained from the American Type Culture Collection. A complete description of the exposure of RAM and CHO cells to particulate samples is provided below.

Treatment of RAM and CHO cells and particulate samples. Macrophages were obtained from male and female New Zealand white rabbits (Myrvik *et al.*, 1961), housed individually and fed antibiotic-free Purina Rabbit Chow and water *ad libitum*. Healthy rabbits weighing 1.5–2.0 kg were sacrificed by injection of sodium pentobarbital (150 mg) into the marginal ear vein; lung lavage *in situ* was performed following irrigation of the neck and chest area with 70% ethanol. Tracheostomies were performed using sterile operating procedures. Thirty milliliters of 0.85% saline (23°C) was introduced into the lungs and allowed to remain for 15 min. After removal of the lavage fluid, five subsequent aliquots were infused and then removed immediately. Lavage fluid found to contain blood or

mucus was discarded. Differential cell counts were performed using a minimum of 200 cells. The average cellular composition of the pooled lavage fluid obtained by counting Wright's stained smears was 97% macrophages, 2% lymphocytes, 0.5% polymorphonuclear leukocytes, and 0.5% eosinophils.

The cells were washed once in normal saline by centrifugation at 450*g* for 15 min at 0–4°C. After a second centrifugation, the cells were resuspended in supplemented Medium 199 with Hanks Salts (0–4°C). The medium was supplemented with 20% heat-inactivated fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), and kanamycin (100 μg/ml) (GIBCO, Grand Island, N.Y.). Cells were counted and viability was determined by means of a hemocytometer.

Particulate samples were initially screened using a concentration of 1000 μg particles/ml culture medium (three to six replicates). The samples were weighed in a vented glove box on a Perkin–Elmer microbalance and added to Falcon 16 × 125-mm test tubes. Particles were vortexed dry for 2 min (setting 8, Vortex Genie) to break up particulate aggregates. Medium 199 (with antibiotics without serum) was added to the particles to bring the concentration to 2000 μg/ml. The suspensions were preincubated for a 20-h period on a Bellco rocker platform (12 oscillations per minute) at 37°C, in a humidified atmosphere containing 5% CO_2 in air.

Preincubated samples were vortexed briefly and 1.0 ml of the suspension was added to each of three wells of Costar six-well cluster dishes. One milliliter of RAM cell suspension (2×10^6 cells/ml) in medium containing 20% FCS was then added to each well containing 1.0 ml of the particulate sample in medium without serum. Cells were dispensed from a spinner flask by means of a Cornwall repetitive syringe. After gentle mixing, the dishes were incubated on a rocker platform for a 20-h period at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Identical procedures were carried out using CHO cells with the exception that the CHO cells were maintained in Ham's F-12 medium supplemented with 10% FCS (virus and mycoplasma screened). The cells were incubated at 37°C in plastic culture flasks and subcultured using 0.25% trypsin twice weekly.

Harvest of cells and cellular and biochemical analysis. After incubation, media containing unattached cells was removed from each dish and maintained separately in an ice bath. Attached cells were removed with 1.0 ml/well of 0.25% trypsin (GIBCO No. 610-5050) and combined with the decanted media. One-tenth milliliter of the combined cell suspension was removed for adenosine triphosphate (ATP) determinations, and 0.5 ml for determination of cell number and viability. One milliliter of the cell suspension was centrifuged at 365*g* for 10 min and resuspended in 3 ml of Dulbecco's phosphate-buffered saline (PBS) at 4°C. These cells were washed again in 3.0 ml PBS. After a third centrifugation, the cells were resuspended in 1.0 ml PBS and an aliquot of 0.5 ml was lysed with 0.5 ml of 2% sodium desoxycholate. The lysate was centrifuged at 12,500 rpm for 3 min with an Eppendorf microcentrifuge to remove particles; 0.25-ml aliquots of the supernatant were analyzed for total protein using bovine serum albumin as a standard (Lowry *et al.*, 1951).

Simultaneous determinations of cell viability and cell numbers were performed. One part of 0.4% trypan blue and five parts of cell suspension were mixed and cell viability was determined by counting a minimum of 400 cells using a hemocytom-

eter. Cell viability was multiplied by total cell numbers as a fraction of the control to yield the viability index (Waters *et al.*, 1975a).

ATP was determined using a Dupont model 760 luminescence biometer according to the following modifications of the procedure (Waters *et al.*, 1975b). Dimethyl sulfoxide (0.4 ml) was used to extract ATP from a 0.1-ml aliquot of trypsinized cell suspension. After 2 min at room temperature, 2.5 ml of cold 0.01 M morpholinopropane sulfonic acid (MOPS) at pH 7.4 was added to buffer the extracted sample. The tube containing the buffered sample was then placed in an ice bath. Aliquots of 10 μl were injected into the luminescence meter's reaction cuvette containing 0.7 mM crystalline luciferin, 100 units luciferase, 0.01 M magnesium sulfate, and 0.01 M MOPS buffer (pH 7.4) at 25°C in a total volume of 100 μl. The emitted light was measured photometrically in the luminescence meter.

Phagocytic activity was measured by addition of 1.1-μm polystyrene latex particles (Dow Diagnostics, Indianapolis, Ind.) to cells (25 particles per cell) cultured in Lab-Tek (Miles Laboratory, Inc., Naperville, Ill.) four-chamber microslides (Waters *et al.*, 1975b). The Lab-Tek slides were seeded with 1.0 ml per chamber of RAM or CHO cells at 5×10^4 cells/ml. (RAM cells were seeded in Medium 199 and CHO cells in Ham's F-12, both media supplemented with 10% FCS and antibiotics.) The slides were incubated for a 2-hr attachment period at 37°C in a humidified atmosphere of 5% CO_2 in air. At the end of the attachment period, 67 μl of 1.1-μm polystyrene latex (1.25×10^6 particles) were added to each well. At various intervals after addition of the latex particles, the slides were drained, air-dried, and exposed for 3 min to concentrated Wright's stain. The slides were then exposed for an additional 5–6 min to a 1:1 aqueous dilution of the Wright's stain. After washing, the slides were air-dried and placed in xylene for 1 hr to dissolve extracellular particles according to the procedure of Gardner *et al.* (1973), and were then mounted in Permount. Phagocytic activity was determined under oil immersion by scoring a minimum of 200 cells. Each cell that contained at least one particle was considered phagocytically active. (The phagocytic activity was considered 100% if all cells contained one or more particles.)

Chinese Hamster Ovary (CHO-K1) Clonal Cytotoxicity Test

The clonal assay using the CHO cell system involved plating of 200–300 cells per 25-cm^2 tissue culture flask (Wininger, 1978). For evaluation of samples, F-12 media were supplemented with 10% FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Four milliliters of CHO cells was added to flasks and the cultures were incubated for 18–24 hr at 37°C to allow for cell attachment. Replicate flasks were exposed to particulate samples in supplemented F-12 media. The cultures were incubated at 37°C with closed caps and allowed to develop discrete colonies of cells. After 6 days the colonies were fixed with 10% formalin in 0.5% NaCl and 4.0% methanol for 30 min and stained with 0.04% crystal violet for 15 min. The flasks were washed with deionized water and allowed to air dry.

Particle Sizing

Sizing analysis was performed using a Coulter counter Model TAII with a 100-μm aperture and counting 20,000 particles. The instrument was calibrated

with 9.69-μm polystyrene latex spheres (Coulter Electronics, Inc.). Filtered (0.45 μm) physiological saline was used as the electrolyte.

Samples (1–2 mg) were weighed into clean polystyrene test tubes, and vortexed 2 min. Saline was added to bring the concentration to 1000 μg/ml. The tubes were then vortexed for 30 sec and set aside. Immediately before counting, the tubes were vortexed for 10 sec and aliquots of 0.1–1.0 ml were added to approximately 100 ml saline and 20,000 particles were counted. Histograms were drawn using the accessory plotter, and both volume and population mean diameter values were determined from cumulative plots (Stockham and Fochtman, 1977).

Experimental Design and Data Analysis

Experiments with either RAM or CHO cells normally consisted of three replicate cultures for each concentration of the test sample and five replicate cultures for control cells. Each experiment was conducted twice; therefore, there was a total of six replicates for experimentals and ten replicates for controls. Experimentals and controls were randomly distributed among the total number of culture dishes. If the initial test of the particle at 1000 μg/ml produced a 50% response, a dose–response curve was obtained in order to estimate the dose level at which the response attained a given magnitude. Samples were tested at concentrations of 10, 50, 100, 250, 500, 750, and 1000 μg/ml, or at some exact multiple of this concentration series.

Data analysis was performed on the cumulative data pool as described previously (Garrett and Stack, 1980).

RESULTS

Phagocytosis of Particulate by RAM and CHO Cells

Particulate matter may exert cytotoxic effects after gaining access to intracellular compartments. The phagocytic activities of RAM and CHO cells exposed to 1.1-μm polystyrene latex spheres were compared; Fig. 1 shows the time courses of phagocytosis. Data in Fig. 1A were obtained when extracellular latex was dissolved with xylene using the method reported by Gardner *et al.* (1973); data in Fig. 1B show the results obtained if the extracellular latex was not dissolved in xylene. The similarity of the curves suggests that particles that are attached to the surface of RAM and CHO cells are rapidly internalized.

The data in Fig. 1 also show that RAM cells were initially the more active phagocytes, but after 8 hr there was no significant difference between the two cell types. The enhanced phagocytic activity of the macrophages during the earlier experimental periods may be related to the RAM's migratory ability, which would allow these cells to contact more spheres. Other experiments showed that both RAM and CHO cells were capable of ingesting latex spheres 5.7 μm and 10.1 μm in diameter. Thus, the RAM and CHO cells were shown to respond like human lung fibroblasts (WI-38) that rapidly phagocytize latex spheres (Garrett *et al.*, 1979).

Since only a nominal difference was observed in phagocytosis of latex particles by xylene-washed and unwashed cells, studies were conducted to determine the attachment and ingestion of particles insoluble in xylene. The attachment and

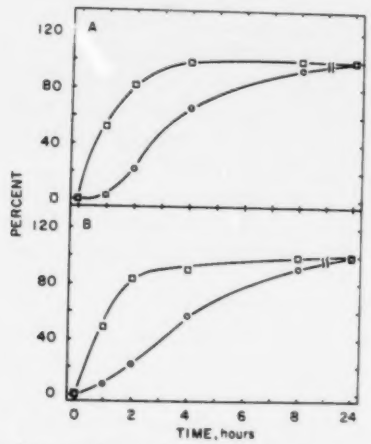

FIG. 1. Uptake of 1.1-μm polystyrene latex spheres by RAM, □, and CHO, ○, cells. A: Extracellular latex was dissolved with xylene. B: Extracellular latex was not dissolved and the curves represent both phagocytized particles and particles attached to the cell surface. The ordinate represents percentage of cells containing one or more particles.

ingestion by RAM cells of samples of fly ash (2–5 μm), relatively inert particles of titanium dioxide (0.45 μm), and fine stack gas particles (<3 μm) are shown in Fig. 2. As can be seen, each of these particle types associated rapidly with the macrophages; the heterogeneity in the particle type and size did not appreciably effect particle uptake. In other experiments CHO cells were also shown to phagocytize fly ash and diesel exhaust particulates (Chescheir *et al.*, 1980).

Internalization of fly ash was confirmed in RAM and CHO cells by light and electron microscopy. Fixed and embedded cells examined by electron microscopy and living cells examined by phase contrast microscopy revealed large numbers of fly ash particles in the cytoplasm.

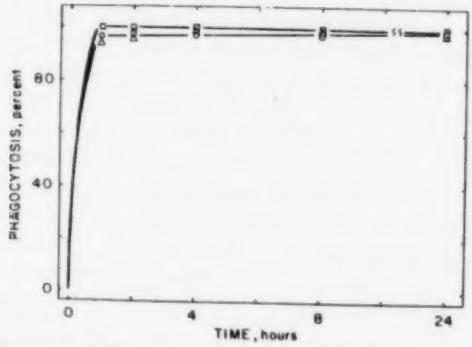

FIG. 2. Rate of attachment and ingestion of stack gas particulate (<3 μm), △, titanium dioxide particles (0.45 μm), □, and fly ash (2–5 μm), ○, by RAM cells. Cells were exposed at a concentration of 250 μg/ml.

TABLE 1
EFFECTS OF CAB-O-SIL (1000 μg/ml) ON RAM AND CHO CELLS

Cell line	Percentage viability	Viability index	ATP[a] (fg/cell)	Cells/ml[a]	Protein[a]
RAM	12.8 ± 2.49	9.14 ± 3.72	2.60 ± 2.34	69.9 ± 19.2	93.9 ± 21.5
CHO	1.70 ± 1.91	0.49 ± 0.55	4.25 ± 5.08	49.7 ± 22.7	67.8 ± 13.7

Note. Average of two experiments (six replicates). The viability of controls was 94.1 and 99.4% for RAM and CHO cells, respectively.

[a] Expressed as a percentage of controls.

Cytotoxicity of Model Compounds

A series of experiments was performed with model substances and particles. The cytotoxicity of these compounds serves as a basis for comparison of the toxicity of particulate materials found in the environment. Toxicity was evaluated in both RAM and CHO cells.

The cytotoxicity of silica (SiO_2) was tested, since coal combustion fly ash contains large quantities of silicon complexes with the particle surface being predominantly aluminosilicate glass (Fisher *et al.*, 1976). Nash *et al.* (1966) previously demonstrated the cytotoxicity of SiO_2 particles.

The cytotoxicity to RAM and CHO cells of Cab-O-Sil (99.8% SiO_2, mesh size 5 μm) after a 20-hr exposure is illustrated in Table 1. Cell ATP and cell viability were sensitive indicators of cellular damage in both cell types. Total cell protein

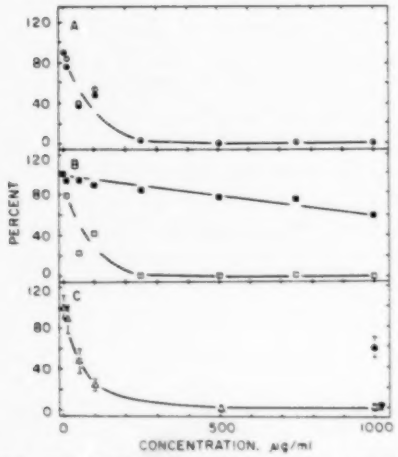

FIG. 3. Cytotoxicity of SiO_2 (Cab-O-Sil) in the RAM (A, B) and CHO (C) cell systems. A: Cell viability, ○, and viability index, ●; B: ATP (fg/cell, percentage of control), □, and cells/ml (percentage of control), ■; and C: CHO colony survival percentage of control, △. In the CHO system washed particles and a filtered supernatant derived by extraction of the particles were also tested. After preincubation of the particles at a concentration of 1000 μg/ml for 20 hr in Ham's F-12 medium with 10% fetal calf serum, the particles were removed by centrifugation and the supernatant passed through a 0.22-μm filter. The washed particles, ▼, were tested at 1000 μg/ml. The filtrate, ◆, was tested undiluted.

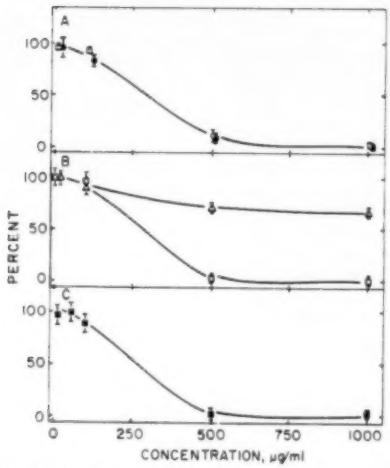

FIG. 4. Cytotoxicity of silicic acid in the RAM (A, B) and CHO (C) cell systems. A: Cell viability, ○, and viability index, ●; B: ATP (fg/cell, percentage of control), □, and cells/ml (percentage of control), △; and C: CHO colony survival, percentage of control, ■. Data are presented as the mean ± standard deviation ($n = 4$ for the RAM assay—no fetal calf serum; $n = 5$ for the CHO assay).

and cell number were significantly depressed for CHO cultures ($P < 0.1$ and $P < 0.001$, respectively). Dose–response curves, obtained from both the short-term RAM assay and the CHO clonal assay, clearly demonstrated the cytotoxicity of SiO_2 as shown in Fig. 3. Cell viability, ATP, and colony survival showed an almost identical response to SiO_2; cell numbers were minimally affected. SiO_2 at a concentration of 100 μg/ml reduced the number of CHO colonies to 24% of the control. No colonies developed when the cells were incubated with either SiO_2 particles or washed SiO_2 particles at 1000 μg/ml. When the supernatant medium derived by extracting SiO_2 particles (1000 μg/ml) was employed, clonal growth was reduced to about 60% of the control. The toxic effects of SiO_2 were easily detected in the RAM assay when separate experiments were conducted using media without FCS. In the RAM assay without serum, 100 μg/ml of SiO_2 particles caused a reduction in viability and ATP to 16.0% and 11.6% of the control, respectively.

TABLE 2
EFFECTS OF TITANIUM DIOXIDE (1000 μg/ml) ON RAM AND CHO CELLS

Cell line	Percentage viability	Viability index	ATP[a] (fg/cell)	Cells/ml[a]	Protein[a]
RAM	91.6 ± 7.57	81.7 ± 17.1	96.2 ± 19.3	88.6 ± 13.1	49.9 ± 23.7
CHO	97.8 ± 2.19	66.5 ± 12.2	106.9[b]	67.9 ± 11.6	87.4 ± 45.4

Note. Average of two experiments (six replicates).
[a] Expressed as a percentage of controls.
[b] Three replicates.

It has been suggested that, in aqueous solution, silicic acid forms the surface component of SiO_2 and acts in hydrogen-bonding reactions with cellular membrane phospholipids (Allison, 1974). The cytotoxicity of silicic acid was measured using both the CHO clonal assay and the short-term RAM assay. The response of RAM and CHO cells to silicic acid was similar to the response obtained for SiO_2 particles. As can be seen in Fig. 4, cell viability, the viability index, and ATP were depressed in the RAM assay to approximately the same extent as was CHO clonal growth. Also, the total number of macrophages was reduced significantly by lysis.

Titanium dioxide particles have been used frequently as "inert particles" in deposition and clearance studies in animals. (See, for example, Ferin and Feldstein, 1978.) TiO_2 particles (0.45 μm) were evaluated for toxicity in both the RAM and CHO cell systems. Results presented in Table 2 show that cell viability is not affected by a 20-hr exposure to a concentration of 1000 μg/ml of particles. The number of CHO cells was significantly less ($P < 0.001$) than the control values, possibly indicating an effect on cell division. In addition, the total protein content of RAM cells was significantly different ($P < 0.001$) from the control.

As part of a study to determine if the overall sensitivity of the short-term assays could be increased by reducing or eliminating serum protein, the toxicity of titanium dioxide particles was evaluated using the macrophage in medium without serum. Results are given in Fig. 5. The most pronounced effect was a decrease in cell ATP; cell number and viability were not strongly affected. Figure 5 also shows the results of particle evaluation in the CHO clonal assay. The number of CHO colonies was about 80% of the control after an exposure to 1000 μg/ml.

Experiments with Model Fly Ash Particles

Fly ash particles with and without coatings of specific metal oxides were obtained from the Illinois Institute of Technology Research Institute. These particles

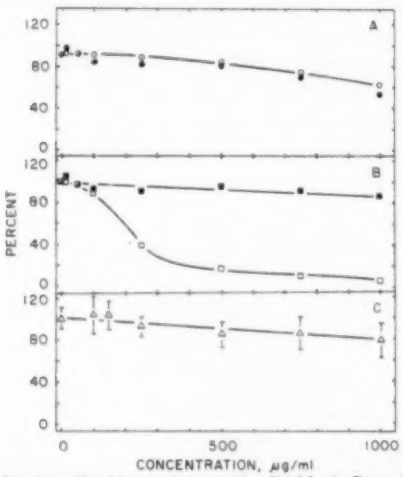

FIG. 5. Cytotoxicity of titanium dioxide particles in the RAM (A, B) and CHO (C) cell systems. A: Cell viability, ○, and viability index, ●; B: ATP (fg/cell, percentage of control), □, and cells/ml (percentage of control), ■; and C: CHO colony survival, percentage of control, △.

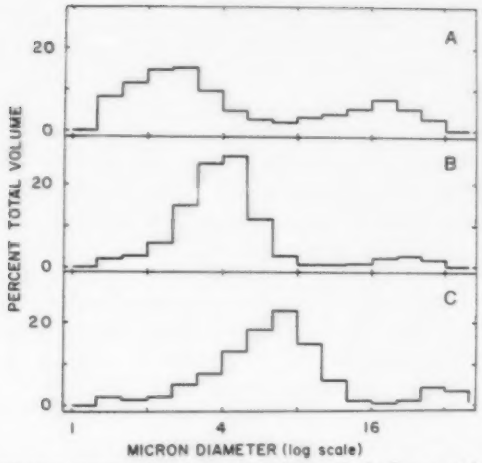

FIG. 6. Size distributions of uncoated fly ash. A: 0–2 μm; B: 2–5 μm; and C: 5–8 μm. Analysis was performed using a Coulter counter as described under Methods.

were collected from an electrostatic precipitator; size-fractionated (designated 0–2, 2–5, 5–8 μm); coprecipitated with lead, nickel, or cadmium; and treated to oxidize the metals (Yamate and Ashley, 1975).

The typical cumulative size distribution analysis of three sizes of uncoated fly ash is shown in Fig. 6. Similar size distribution patterns were obtained from the metal-oxide coated fly ashes. The volume mean diameters of the particles were calculated from the size distribution patterns (Stockham and Fochtman, 1977). These data are shown in Table 3. Population mean diameters are also given for some of the particles. The data from the distribution analysis show a broad range of particle sizes. Although the smaller particle fraction (0–2 μm) contained what may be larger particle aggregates and the largest particle fraction (5–8 μm) was severely contaminated with smaller particles, the mean diameters agreed well with the original size fractions.

The uncoated control fly ash (2–5 μm) was found to be relatively nontoxic to

TABLE 3
PARTICLE SIZE MEAN DIAMETER OF FLY ASHES

	0–2 μm VMD (PMD)	2–5 μm VMD (PMD)	5–8 μm VMD (PMD)	5–10 μmf VMD (PMD)
PbO coated	3.40 (1.82)	4.25 (2.95)	6.8 (4.73)	
NiO coated	3.60	6.90	9.30	
	3.90	5.70	6.65	
CdO coated	3.45	5.10		8.70
Uncoated	3.00	4.10	6.50 (3.90)	6.30

Note. VMD = Volume mean diameter; PMD = population mean diameter.

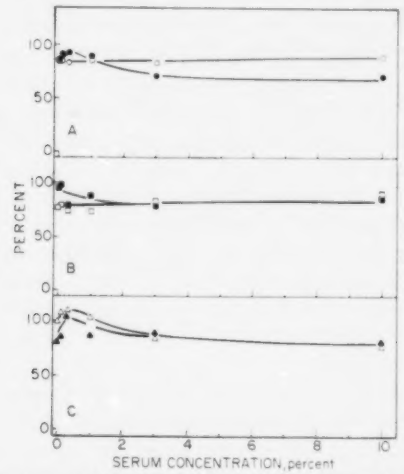

FIG. 7. Influence of serum concentration on the RAM bioassay of model fly ash (0–2 μm). A: Cell viability, ○, and viability index, ●; B: ATP (fg/cell, percentage of control), □, and ATP (fg/mg protein, percentage of control), ■; and C: cells/ml, percentage of control, △, and cell protein, percentage of control, ▲. Cells were exposed for 20 hr to 1000 μg/ml fly ash. Data points are mean values of three replicates.

RAM cells, as can be seen in Fig. 7. After a 20-hr exposure, cell number, viability, and cell ATP in cultures treated with 1000 μg/ml particle were not significantly different from control values. In addition, no appreciable difference in toxicity was observed when the serum content of the media was reduced to zero.

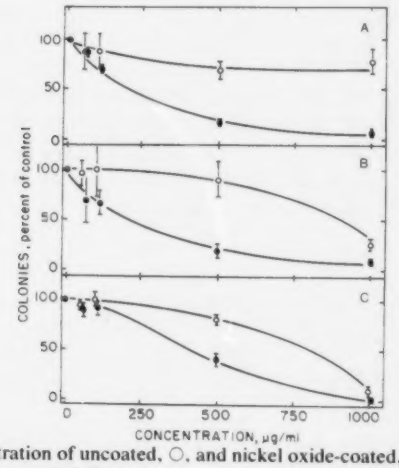

FIG. 8. Effect of concentration of uncoated, ○, and nickel oxide-coated, ●, fly ash on CHO clonal growth after a 6-day exposure period. Data are presented as mean ± standard deviation (n = 5). Panel A represents results for 5- to 8-μm particles; Panel B for 2- to 5-μm particles, and Panel C for 0- to 2-μm particles.

In order to further examine the toxicity of uncoated fly ash, experiments were conducted using the CHO clonal assay (Fig. 8). Fly ash without a metal oxide coating was relatively nontoxic at concentrations less than 500 μg/ml. The number of colonies formed was reduced at a concentration of 1000 μg/ml to 10.6 and 28.5% of the control for the 0 to 2-μm and 2 to 5-μm size particles, respectively. The 5- to 8-μm uncoated fly ash was relatively nontoxic (82.2% of the control) at 1000 μg/ml, possibly due to decreased phagocytosis of this size particle.

Figure 8 also shows that NiO-coated fly ash was more toxic than the uncoated particles for all three particle sizes. The approximate nickel content of the particles determined by atomic absorption was 2.6 to 2.9% (Aranyi, 1976; Aranyi *et al.*, 1977). Nickel was tightly bound to the fly ash and did not dissociate appreciably from the particles at physiological pH. Free nickel was measured using atomic absorption spectrophotometry.

The relative toxicities of NiO-coated fly ash (0–2 μm), NiO, $NiCl_2$, and Ni_3S_2 were examined in the CHO clonal system. Table 4 shows that the toxicity of the NiO-coated fly ash was similar to that of NiO. The cloning efficiency of CHO cells was extremely sensitive to $NiCl_2$ and Ni_3S_2, both of which have been shown to be carcinogenic in several *in vivo* and *in vitro* bioassays (Schroeder, 1971).

The NiO-coated particles were also more toxic than the uncoated particles in the RAM system as can be seen in Fig. 9. Cell ATP, viability, and protein were significantly different ($P < 0.001$) from the controls at a concentration of 1000 μg/ml.

Lead oxide-coated particles (3.2 to 3.9% metal content; Aranyi, 1976; Aranyi *et al.*, 1977) were used to further examine the influence of serum on the various endpoints studied in the RAM assay. As in the case with nickel, lead was determined by atomic absorption spectrometry to be tightly bound to the fly ash and did not dissociate from the particles into the medium. As can be seen in Fig. 10 the lead oxide-coated particles were significantly more toxic than nickel oxide-coated particles (see Fig. 9 data points at 1000 μg/ml) at a 10% serum concentration. Cell ATP, viability, and viability index decreased as the serum content of the culture medium was reduced. ATP was the most sensitive measure of toxicity; however, cell viability and the viability index were sharply reduced at serum concentrations less than 1%. Protein and cell number as percentages of the control were not appreciably affected. Cell viability and ATP did not change significantly in control cells as the serum concentration was decreased.

TABLE 4

RELATIVE TOXICITY (PERCENTAGE SURVIVING COLONIES) OF NiO-COATED FLY ASH, NiO, $NiCl_2$, AND Ni_3S_2 IN THE CHO CLONAL ASSAY

Sample	Concentration (μg/ml)					
	Control	10	50	100	500	1000
NiO-coated fly ash (0–2 μm)	100 ± 5.84	91.4 ± 10.3	90.0 ± 6.54	91.5 ± 8.30	42.1 ± 4.82	2.18 ± 0.31
NiO	100 ± 7.14	95.9 ± 10.1	83.2 ± 15.6	84.6 ± 18.5	54.0 ± 32.0	10.7 ± 11.2
$NiCl_2$	100 ± 7.14	94.0 ± 6.8	12.3 ± 13.6	0.0	0.0	0.0
Ni_3S_2	100	44.4	0.0	0.0	0.0	0.0

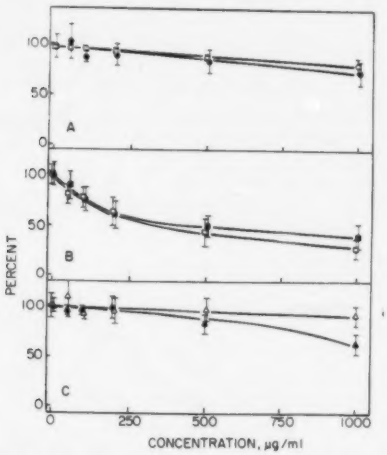

FIG. 9. Effect of concentration of nickel oxide-coated fly ash (<2 μm) on RAM cells after a 20-hr exposure. Data are presented as mean ± standard deviation for three experiments (n = 9). A: Cell viability, ○, and viability index, ●; B: ATP (fg/cell, percentage of control), □, and ATP (fg/mg protein, percentage of control), ■; and C: cells/ml, percentage of control, △, and cell protein, percentage of control, ▲.

Data plotted in Fig. 11 show the absolute values of ATP and protein per cell for RAM cultures treated with PbO-coated and uncoated fly ash (both <2 μm). Protein per cell (Fig. 11A) was reduced as the serum content was lowered below 3% in the control or in cultures exposed to either coated or uncoated particles. Protein/cell was substantially lower in cultures treated with PbO-coated particles. ATP/cell (Fig. 11B) did not vary significantly with serum concentrations in controls or cultures treated with uncoated particles; but, ATP was strongly affected in cul-

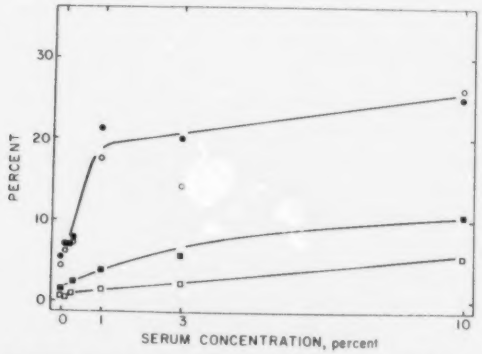

FIG. 10. Influence of serum concentration on cell viability, ○, viability index, ●, and ATP, □ (fg/cell, percentage of control) or ■ (fg/mg protein, percentage of control) in the RAM assay. Cells were exposed to lead oxide-coated fly ash (<2 μm) for 20 hr at a concentration of 1000 μg/ml. Data points are mean values of three replicates.

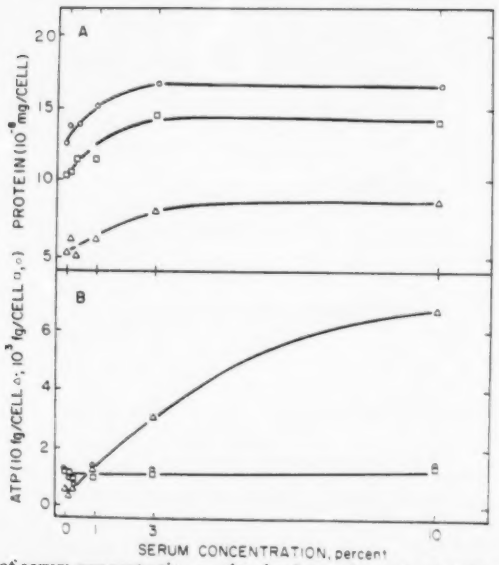

FIG. 11. Influence of serum concentration on the absolute values of cell ATP and total protein. The response of RAM cells was measured after a 20-hr exposure to 1000 μg/ml (<2 μm) of uncoated, □, and lead oxide-coated fly ash, △. Data points are mean values of three replicates for treated cells and six replicates for the control, ○. (A) Protein data, (B) ATP data.

tures treated with PbO-coated particles. The cellular ATP content was about 100 times less in the metal oxide-treated cultures and ranged from 20 to 200 times less than that for the uncoated fly ash or the control.

Experiments with CdO-coated fly ash proved particularly interesting since cadmium was found to dissociate from the particles into the biological medium. The cadmium content of the particles was 11.9 to 12.3% (Aranyi, 1976; Aranyi *et al.*, 1977). Dissociation of cadmium from the coated fly ash was determined by atomic absorption analysis and is shown in Fig. 12A. Soluble cadmium was removed by centrifugation of the particles after a 2-hr preincubation period. The time course for toxicity of the CdO-coated particles to RAM cultures was then determined; results are given in Figs. 12B, C.

The rate of dissociation of the metal from the washed particle (Fig. 12A) correlated well with the loss of ATP (Fig. 12C). ATP, as a percentage of the control, decreased more rapidly with increasing exposure times than did the cell viability or the viability index.

After integration of the area under the curve describing release of cadmium, the average exposure of soluble cadmium was calculated over a 20-hr period for a unit amount of CdO-coated fly ash. The EC_{50} for the CdO-coated particles was approximately 30 μg/ml (Fig. 13), which corresponded to an effective 20-hr exposure of about 6 μM of soluble cadmium. The published value of the EC_{50} for soluble cadmium in the RAM assay is 79 μM (Huisingh *et al.*, 1977). Thus, CdO-coated fly

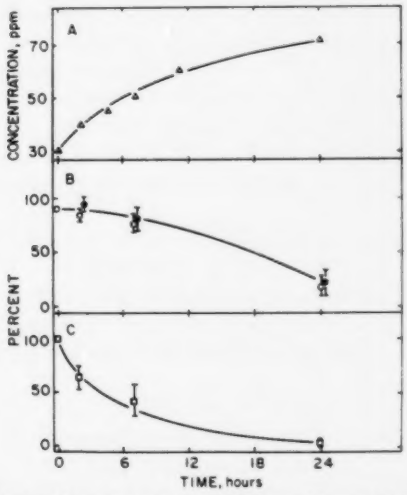

FIG. 12. Release of cadmium from 0- to 2-μm cadmium oxide-coated fly ash (A) and the effect of concentration of the particles on RAM cells (B, C). A: ppm cadmium, △; B: cell viability, ○, and viability index, ●; and C: ATP (fg/cell, percentage of control), □. Dissociation of cadmium into the medium from 1120 μg/ml of particulate was measured by atomic absorption analysis. RAM cells were exposed to 60 μg/ml of the particles (mean ± standard deviation; $n = 6$).

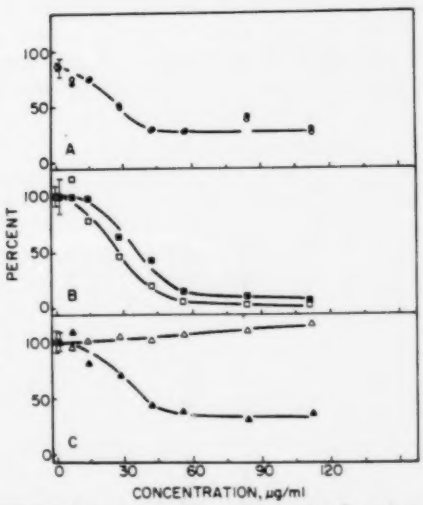

FIG. 13. Effect of concentration of cadmium oxide-coated fly ash on RAM cells after a 20-hr exposure. Data points are mean values of three replicates for treated cells and six replicates for the control. A: Cell viability, ○, and viability index, ●; B: ATP (fg/cell, percentage of control), □, and ATP (fg/mg protein, percentage of control), ■; and C: cells/ml, percentage of control, △, and cell protein, percentage of control, ▲.

ash was considerably more toxic than would have been predicted on the basis of the amount of soluble cadmium released into the medium.

Additional evidence that cadmium rapidly dissociated from the particles was obtained by incubation of macrophages with the briefly washed fly ash under the usual conditions. Dose–response curves are given in Fig. 13. Cell viability and the viability index decreased sharply with ATP and total protein; but, cell numbers were unaltered as has been reported previously with soluble cadmium (Waters *et al.*, 1975a, 1978).

The biological responses of CHO and RAM cells to the CdO-coated fly ash were also compared. Figure 14 shows that the CHO cells responded similarly to the RAM cells. RAM and CHO data were especially alike with respect to the viability index and ATP/mg protein. Cell number was lower in CHO cultures treated with the CdO-coated fly ash suggesting that the particles inhibited cell division.

DISCUSSION

The cytotoxicity of mineral dusts and fibers has been studied extensively in short-term cultures of macrophages and continuous cell lines (Harington, 1976; Harington *et al.*, 1975; Chamberlain and Brown, 1978). Christian and co-workers (1978, 1979) have also investigated the effects of leachates of coal dust on the growth of mammalian cells and the results paralleled epidemiological evidence of coal workers' pneumoconiosis. Nonetheless, cell systems have not been used extensively to evaluate the toxicity of airborne particles such as those emitted into the environment during energy conversion processes. Experimental data reported here detail the effects of model particulate material and fly ash particles on RAM and CHO cells.

RAM and CHO cells were both found to be capable of phagocytizing a variety of particulate matter including fly ash from coal combustion. An early, enhanced

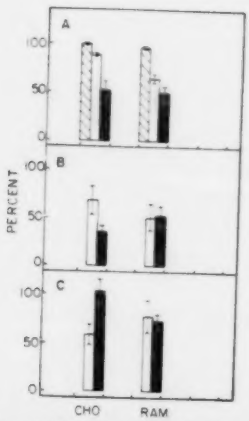

FIG. 14. Effect of 30 μg/ml cadmium oxide-coated fly ash (< 2 μm) on CHO and RAM cells. Data are presented as mean ± standard deviation (n = 4). A: Cell viability, □, viability index, ■, and control viability, ▨; B: ATP (fg/cell percentage of control), □, and (fg/mg protein, percentage of control), ■; and C: cells/ml, percentage of control, □, and cell protein, percentage of control, ■.

phagocytic activity of the RAM was noted that may be related to the migratory ability of this cell type. Evidence was also obtained that particles which are attached to the surface of RAM and CHO cells are rapidly internalized.

Since fly ash particles contain large quantities of metal–silica complexes, the toxicity of silica was evaluated in the RAM and CHO cell systems. Previous studies have shown that the cytotoxicity of silica particles is due to the action of hydrolases released into the cell after damage to the lysosomal membrane (Nash *et al.*, 1966; Nadler and Goldfischer, 1970). Most crystalline forms of silica are hemolytic and cytotoxic and stimulate a fibrogenic reaction in the lung (Allison, 1974). Our experiments showed that cell viability and ATP in the short-term RAM assay, with and without serum, and cell cloning in the CHO assay are effective indicators of the cytotoxicity of silica. Similar results were obtained for silicic acid which may form the surface component of silica (Allison, 1974).

Titanium dioxide, a relatively inert particle, was also evaluated in the RAM and CHO test systems. Electron microprobe analysis has shown that TiO_2 is found as a minor component of the surface of fly ash particles (Fisher *et al.*, 1976). Although the particles were found to be considerably less toxic than other particulate compounds, ATP was sharply reduced when cells were exposed in serum-free media to TiO_2 particles. Since 100 million pounds of TiO_2 are estimated to be released into the U.S. atmosphere annually (Schroeder, 1971), these results suggest that the possible toxicity of titanium particles should be further studied.

Toxic or carcinogenic trace elements (e.g., Pb, Cd, Se, As, Ni, and Cr) have been shown to be enriched in the finest particles emitted from a coal-fired power plant (Davison *et al.*, 1974). Studies of the toxicity of model particles coated with specific metal oxides were conducted in the RAM and CHO cell systems to provide an indication of the ability of these short-term assays to assess the hazard presented by atmospheric particulate matter produced by coal combustion processes.

Aranyi *et al.* (1977, 1979) have studied RAM cell viability and enzyme levels after exposure of cells to the nondissociable metals Pb, Mn, and Ni adsorbed on fly ashes. These investigators established that lactic dehydrogenase levels but not lysosomal acid phosphatase or β-glucuronidase were decreased with increasing concentrations of the particles suggesting that the loss of soluble cell components was a primary event in the cytotoxic response.

In our experiments uncoated fly ash was relatively nontoxic in the RAM assay when determined by measurement of cell number and viability, ATP, and protein. Only small differences in toxicity were observed for these particles when serum was removed from the media. The CHO clonal assay provided a more sensitive test for the toxicity of uncoated fly ash. In this assay, the smaller particles were the most toxic as has been shown using the macrophage assay (Aranyi *et al.*, 1977, 1979).

The toxicity of NiO-coated fly ash is of some importance, since combustion of fuel oil and coal is a major contributor to atmospheric nickel (Schroeder, 1971). Nickel oxide-coated fly ash was found to be more toxic than the uncoated particles for all three particle sizes in both the RAM and CHO assay. The CHO system was sensitive to NiO as well as to $NiCl_2$ and Ni_3S_2. At equivalent concentrations, the toxicity of NiO was similar to that observed for NiO-coated fly ash although

the weight percentage of NiO in the ash was only 3%. Since the uncoated particles were nontoxic in the CHO system for concentrations less than 500 μg/ml, these data indicate that the association of the metal oxide with the particle enhances cytotoxicity. However, the similar toxicity of NiO and NiO-coated fly ash could alternatively be explained if toxicity is primarily a result of the interactions of the cells with the particle surface.

Lead is also a serious elemental pollutant of the atmosphere and is of special significance since the lead ion inhibits a large variety of cellular enzymatic reactions, and produces damage to the bone marrow, kidney, liver, and the reproductive and central nervous systems (Grandjean, 1978). Lead oxide-coated particles were tested to provide additional information concerning the influence of serum concentration on toxicity determined in the macrophage assay. The PbO-coated particles were found to be significantly more toxic than either the uncoated or NiO-coated particles. Although cell viability after exposure to the particles was sharply reduced at serum concentrations less than 1%, cell ATP was found to be the most sensitive indicator of toxicity. At 1% serum, ATP/cell was about 100 times less in the PbO-treated cultures. The ATP/cell value ranged from approximately 20 to 200 times less than that for uncoated fly ash or the control. These experiments tend to confirm the previously reported differential effects of serum protein on the cytotoxicity of mineral particles. (Harington, 1976; Harington *et al.*, 1975). High serum concentrations have also been reported to decrease the toxic effect of coal dust leachate (Christian *et al.*, 1979). Additional discussion of the effects of serum on the cytotoxicity of particles from coal-related processes is given in the accompanying paper (Garrett *et al.*, 1981).

Since the RAM assay was originally conceived as a system that should respond to toxic components that dissociate from the surface of respirable particles, cadmium oxide-coated fly ash was especially suitable as a particulate model. Cadmium was found to dissociate rapidly from the coated particle. The CdO-coated fly ash also proved to be considerably more toxic than would have been predicted on the basis of the amount of soluble cadmium released into the medium. Similar data were obtained when CHO cells were exposed to the CdO-coated fly ash. This finding may be important to future considerations of the potential toxicity of dissociable metals that adsorb to the surface of particles released from high-temperature combustion processes. Related studies have been reported (Lee and von Lehmden, 1973; Natusch *et al.*, 1974; Natusch, 1978).

In summary, the studies reported here have been aimed at the evaluation of the RAM and CHO cell systems for assay of the toxicity of airborne particles. RAM and CHO cells phagocytize a variety of particulate matter and the effects of these particles on cell viability or ATP in RAM or CHO cells seems to be closely correlated with their effects on CHO cell cloning. In additonal studies (Garrett *et al.*, 1981) we were able to show that the RAM system without serum was about equivalent in sensitivity to the CHO cloning assay for particles derived from coal-related processes. These *in vitro* studies of cytotoxicity at the cellular level can be considered a reasonable first step in the biological evaluation of toxic particulate substances. The data presented here show that the RAM and CHO cell systems are both sensitive to toxic particulate materials and that these systems

may be used to detect and possibly rank the toxicity of particles obtained from various stages of industrial and energy conversion processes.

ACKNOWLEDGMENT

This work was carried out under Environmental Protection Agency Contract 68-02-2566 to Northrop Services, Inc. The authors are grateful to Dr. Ray Merrill and Mr. Bill Rhodes and Mr. Bruce Henschel of the Process Measurements Branch, Industrial Environmental Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, N.C., for reviewing the manuscript.

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